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(54) Title: ANTIANGIOGENIC DRUGS

(57) Abstract

The invention relates to the use of peptides individually or in combination, for treating and/or preventing angiogenesis. It also relates to the use of peptide analogs or a combination of peptides referred to as MuJ–7 as anticancer drugs in restricting the tumor growth and spread by inhibiting tumor angiogenesis. MuJ–7, in addition inhibits metastasis through its antiangiogenic activity in all cancers. The invention also relates to a pharmaceutical composition containing either individual peptides or in combination, and methods of treatment of human beings and animals for curing and/or preventing angiogenesis.

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ANTIANGIOGENIC DRUGS

The invention relates to the use of peptides individually or in combination, for treating and/or preventing angiogenesis. It also relates to the use of a combination of peptides referred to as MuJ-7 as an anticancer drug in restricting tumor growth and its spread by inhibiting tumor angiogenesis. MuJ-7, in addition inhibits metastasis through its antiangiogenic activity in all cancers. The invention also relates to a pharmaceutical composition containing either individual peptides or combinations of peptides, and methods of treatment of human beings and animals for curing and/or preventing angiogenesis.

Angiogenesis is the growth of new microvessels. This process depends mainly on locomotion, proliferation, and tube formation by capillary endothelial cells. During angiogenesis, endothelial cells emerge from their quiescent state and can proliferate as rapidly as bone marrow cells, but unlike the bone marrow, angiogenesis is usually focal and of brief duration. Pathologic angiogenesis, while still a focal process, persists for months or years. The angiogenesis that occurs in diseases of ocular neovascularisation, arthritis, skin diseases, and tumors rarely terminates spontaneously and has until recently, been difficult to suppress therapeutically. Therefore, the fundamental goal of all antiangiogenic therapy is to return foci of proliferating microvessels to their normal resting state, and to prevent their regrowth[1].

Although the molecular mechanisms responsible for transition of a cell to angiogenic phenotype are not known, the sequence of events leading to the formation of new vessels has been well documented[2,3]. The vascular growth entails either endothelial sprouting[3,4] or intussusception[5]. In the first pathway, the following sequence of events may occur: (a) dissolution of the basement of the vessel, usually a postcapillary venule, and the interstitial matrix; (b) migration of endothelial cells toward the stimulus; (c) proliferation of endothelial cells trailing behind the leading endothelial cell(s); (d) formation of lumen (canalization) in the endothelial array/sprout; (e) formation of branches and loops by confluence/anastomoses of sprouts to permit blood flow; (f) investment of the vessel with pericytes; and (g) formation of basement membrane around the immature vessel[2,3]. New vessels can also be formed via the second pathway: insertion of interstitial tissue columns into the lumen of preexisting vessels. The subsequent growth of these columns and their

stabilization result in partitioning of the vessel lumen and remodelling of the local vascular network[5,6].

The rationale for antiangiogenic therapy is that progressive tumor growth is angiogenesis-dependent[8]. The switch to the angiogenic phenotype appears to be an independent event that occurs during the multistage progression to neoplasia[9]. The angiogenic switch itself, while relatively sudden and well localized, is nonetheless a complex process. This phenotype is currently understood in terms of a shift in the net balance of stimulators and inhibitors of angiogenesis, during which inhibitors are down regulated[10,11].

Once new capillary loops converge toward a small in situ carcinoma or a microscopic metastasis, the tumor cells are bathed in additional survival factors and growth factors, not only from the circulating blood (perfusion effect) but also from vascular endothelial cells themselves (paracrine effect). The positive regulators of angiogenesis include at least 14 angiogenic proteins that have been discovered during the past 12 years and which have been sequenced and cloned[12]. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the most well studied and are found in a majority of different types of human tumors. During the angiogenic switch one or more of these angiogenic stimulators is upregulated and it appears, however, that this up regulation of angiogenic stimulators is accompanied by down regulation of local tissue inhibitors of angiogenesis.

The paracrine stimulation of tumor cells by products from endothelial cells also operates in the other direction. Endothelial cell survival and growth are driven by tumor derived mitogens and motogens. These findings have led to a model of tumor growth in which the endothelial cell compartment and the tumor cell compartment interact with each other. They not only stimulate each other's growth, but if the endothelial cells are made unresponsive to angiogenic stimuli from the tumor cells, by administration of a specific endothelial inhibitor, both primary tumors[11] and metastatic tumors[10] can be held dormant, at a microscopic size. One could take advantage of this difference between endothelial cells and tumor cells by administering an angiogenesis inhibitor together with conventional cytotoxic chemotherapy up to the point at which the cytotoxic therapy would normally be discontinued because of toxicity or drug resistance. The angiogenesis inhibitor(s)

could then be continued (for years), to maintain either stable disease or tumor dormancy[1]. Such combinations of antiangiogenic and cytotoxic therapy in tumor-bearing animals have been curative, whereas either agent alone is merely as inhibitor[15].

Peptides/Proteins have previously been studied for antiangiogenic activity. Thrombospondin- 1 (TSP-1) which is a naturally occurring inhibitor of angiogenesis, makes endothelial cells unresponsive to a wide variety of inducers. Both native TSP-1 and small antiangiogenic peptides derived from it show that this inhibition is mediated by CD36[16] (Dawson et al., 1997). Both IgG antibodies against CD36 and glutathione -S-transferase-CD36 fusion proteins that contain the TSP-1 binding site blocked the ability of intact TSP-1 and its active peptides to inhibit the migration of cultured microvascular endothelial cells.

The family of tissue inhibitors of metalloproteinases (TIMPs) are known to be specific inhibitors of matrix metalloproteinases (MMPs). The local balance between MMPs and TIMPs is believed to play a major role in extracellular matrix (ECM) remodelling during diseases such as cancer. TIMP-3 which is unique in being a component of ECM, inhibits endothelial cell migration and tube formation in response to angiogenic factors[17] (Anand-Apte et al., 1996).

The conditioned medium of human promyelocytic leukemia (HL6O) cells has been shown to contain a cell growth inhibitory factor, human cytostatin. Human cytostatin can inhibit endothelial cell proliferation, migration and microvessel tube formation on Matrigel-coated surfaces[18] (Yeung AK et al., 1996) . Furthermore the anti-angiogenic effect of human cytostatin has been demonstrated on the chick chorioallantoic membrane. Human cytostatin can inhibit new blood vessel development, but cannot regress existing blood vessels.

Angiostatin, which is a 38 kD internal fragment of plasminogen is an antiangiogenic endothelial cell inhibitor and suppresses the growth of primary Lewis lung carcinoma *in vivo*[19] (Wu, Z. et al., 1997).

Thalidomide has recently been shown to antagonize basic fibroblast growth factor-induced angiogenesis in the rat corneal micropocket assay. It has been suggested that thalidomide elevates tumor hypoxia in the Lewis lung tumor, presumably via an antiangiogenic mechanism[20] (Minchinton AI et al., 1996).

One study examined the *in vitro* antiangiogeneic effects of the somatostatin analog octreotide on the growth of human umbelical vein endothelial cells (HUVEC) and vascular cells from explants of rat aorta cultured on fibronectin-coated dishes or included in fibrin gel. A total 10^{-9}M octreotide reduced the mean uptake of ^{3}H-thymidine by HUVEC cells by 37% compared with controls. The 10^{-8M} concentration of octreotide inhibited the proliferation of endothelial and smooth muscle cells growing on fibronectin by 32.6% and reduced the sprouting of cells from the adventitia of aortic rings in fibrin by 33.2% compared with controls, as measured by tetrazolium bioreduction and image analysis, respectively. These results demonstrate that octreotide is an effective inhibitor of vascular cell proliferation *in vitro*[21] (Danesi R. Del Taccam, Metabolism 1996. Aug. 45(8 Supp 1: 49-50) . In another experiment, somatostatin analogs SMS 201-995 and RC- 160 were found to inhibit angiogenesis in the chick chorioallantoic membrane of the developing chicken embryo. Somatostatin analogues were associated in a dose-related fashion with both a greater percentage of inhibition of blood vessel growth and an increased grade of inhibition. It was hypothesized that inhibition of angiogenesis may be a mechanism responsible for the tumor regression observed *in vivo* following SMS or RC-160 therapy[22] (Woltering EA et al., J. Surg. Res. March 1991, 50(3): 245-251).

The proinflammatory neuropeptide, substance P, stimulated angiogenesis in an *in vitro* model using HUVECs cultured on a basement membrane (Matrigel) substrate. Substance P stimulated endothelial cell differentiation into capillary-like structures in a dose-dependent manner. Stimulation of endothelial cell differentiation is a newly recognized biological function of substance P[23] (Wiedermann, C.J. et al. Eur. J. Pharmacol. March 18, 1996, 298 (3): 335-338).

The effects of Nitric oxide (NO) generation and endogenous production of NO elicited by substance P (SP) in angiogenic process were evaluated in the rabbit cornea (*in vivo*) and by measuring growth and migration of endothelial cells (*in vitro*). The NO synthase inhibitors given systemically inhibited angiogenesis elicited by [sar9]-SP-Sulfone. Capillary endothelial cell proliferation and migration produced by SP were abolished by pretreatment with the NO synthase inhibitors. Exposure of the cells to SP activated the calcium-dependent NO synthase. These data indicate that NO production induced by vasoactive agents, such as SP, functions as an

autocrine regulator of the microvascular events necessary for neovascularization and mediates angiogenesis[24] (Ziche M. et al., J. Clin. Invest. Nov. 1994, 94(5):2036-2044).

The angiogenic activity of four vasoactive peptides with a range of vasodilator and vasoconstrictor properties were investigated in a rat sponge model. 2 daily doses of vasodilator peptide VIP(10 pmol) when given with interleukin-1 alpha caused intense neovascularization which was inhibited by simultaneous administration of VIP(10-28), a specific VIP receptor antagonist. These data show that VIP possesses angiogenic activity and the blockade of VIP induced angiogenesis at the receptor level could provide a strategy for the management of angiogenic disease[25].

Vascular endothelial cells are important in a variety of physiological and pathophysiological processes. The growth and functions of vascular endothelial cells are regulated both by soluble mitogenic and differentiation factors and by interactions with the extracellular matrix; however, relatively little is known about the role of the matrix. The neuropeptide bombesin, the bioactive lipid lysophosphatidic acid (LPA), and the cytokine tumor necrosis factor alpha, which signal through diverse mechanisms, were all able to activate MAPK to a much greater degree in fibronectin adherent cells than in suspended cells. Together, these data suggest a cooperation between integrins and soluble mitogens in efficient propagation of signals to downstream kinases. This cooperation may contribute to anchorage dependence of mitogenic cell cycle progression (Short SM et al, Molecular Biology of the Cell 9(8):1969-1980, 1998).

Cell adhesion to the extracellular matrix (ECM) has been implicated in apoptosis in anchorage-dependent cell types. It was recently found that a peptide derived from fibronectin (termed III14-2) inhibits the integrin-mediated cell adhesion to ECM. Using this antiadhesive peptide and a variety of ECM proteins, a critical role of the integrin-ECM protein interaction in apoptotic regulation of human umbilical vein endothelial cells (HUVEC) has been demonstrated. HUVEC in suspension undergoes apoptosis under the serum-free conditions, as judged by nuclear and DNA fragmentations (Fukai F et al, Experimental Cell Research, 242(1):92-99, 1998).

It has been previously shown that vasoactive intestinal polypeptide (VIP) induces endothelium-dependent relaxation of the human uterine artery. Non-

competitive antagonism with methylene blue revealed that the pKa value for VIP-receptor complex was 8.10+/-0.10 (n=6) and the receptor reserve expressed as K-A/EC50 was 0.89+/-0.11, where pKa=log (10)K(A), and K-A is the dissociation constant of VIP-receptor complex (Jovanovic A. et al. Molecular Human Reproduction, 4(1):71-76, 1998).

Somatostatin (SRIF) exerts antiproliferative effects, and has recently been evaluated in clinical trials for the prophylaxis of restenosis following coronary angioplasty. 3 SRIF (0.1-1000 nM) caused a concentration-dependent inhibition of the bFGF-stimulated regrowth in CHO-K1 cells expressing human sst(2) (h sst(2)) or sst(5) (h sst(5)) receptors (pIC(50)=8.05+/-0.03 and 8.56+/-0.12, respectively). SRIF (0.1-1000 nM) was able to inhibit the bFGF-stimulated re-growth (pIC(50)=7.98+/-24 and 8.50+/-0.12, respectively). (Alderton F et al, British Journal of Pharmacology, 124(2): 323-330, 1998).

Substance P (SP) was analyzed in rat brain endothelium cultures after cytokine stimulation. SP secretion was found after stimulation with high doses of interleukin-1 beta (IL-1 beta) and tumor necrosis factor alpha (TNF-alpha). Under cytokine stimulation, part of SP was bound to brain endothelial cell surface, suggesting the existence of an autocrine network for this neuropeptide. SP regulates cellular processes in the CNS, placenta and vasculature include permeability, inflammation, mitogenesis and transformation. Increased SPR mRNA level in response to E-2 were linearly related to increased [H-3]SP binding to the SPR (Villablanca AC et al, Molecular & Cellular Endocrinology, 135(2):109-117, 1997).

The present invention provides pharmaceutical compositions for treating cancer angiogenesis and cancer metastasis. The invention provides a method of treating angiogenesis, cancer and cancer metastasis employing a pharmaceutically effective dosage of a combination of peptides or individual peptides. It is an object of this invention that a combination of peptides used is known as MuJ-7. Individual constituent peptides of MuJ-7 and pharmaceutically acceptable additives can be used. The present invention provides a pharmaceutical composition useful for killing or inhibiting multiplication of tumor cells as well as cancer cells. The pharmaceutical composition may also be useful in preventing, inhibiting, or modulating the hypersecretion of VIP, somatostain, bombesin, Substance P, or a combination of VIP,

somatostatin, bombesin, or Substance P.

The composition may suitably comprise, consist of, or consist essentially of a therapeutically effective combination of peptide analogs of somatostatin, VIP, bombesin, and Substance P. The peptide analogs are described in more detail below, but constituents functionally interchangeable with those specifically described may also be employed in the claimed pharmaceutical composition. More particularly, the pharmaceutical composition may suitably comprise, consist of, or consist essentially of an analog of somatostatin and at least four peptides selected from the group consisting of a first analog of VIP, a second analog of VIP, a third analog of VIP, analog of somatostatin another analog of somatostatin, an analog of bombesin, and an analog of Substance P. More particularly, the composition may suitably comprise, consist of, or consist essentially of a therapeutically effective combination of peptide SOM_2 (an analog of somatostatin) and at least four of the following peptides: VIP_1 (a VIP anatagonist), VIP_2 (a VIP) receptor binding inhibitor), VIP_3 (a VIP receptor antagonist), SOM_1 (a somatostatin analog (also abbreviated "CTOP." which is derived from the first letters of the following four amino acids: Cys^2, Tyr^3, Orn^5, and Pen^5), BOM_1 (a bombesin antagonist), and SP_1 (a Substance P antagonist). In a preferred embodiment, a pharmaceutically acceptable carrier, diluent, or solvent is used. The invention provides a method of treatment for humans, mammals, or other animals suffering from cancer or other tumors. The invention also provides a method of treatment for humans, mammals, or other animals suffering from hypersecretion of VIP, somatostatin, bombesin, Substance P, or a combination of VIP, somatostatin, bombesin, or Substance P. The method may suitably comprise, consist of, or consist essentially of administering a therapeutically effective dose of the pharmaceutical composition so as to prevent, inhibit, or modulate the hypersecretion of VIP, somatostatin, bombesin, Substance P, or a combination of VIP, somatostatin, bombesin, or Substance P.

In addition, the compositions may comprise, consist essentially of or consist of one or more of peptides identified below as DT-11; DT-12; DT-13; DT-14; DT-15; DT-16; DT-18; DT-19; DT-23; DT-24; DT-26; DT-27; DT-31; DT-33; DT-34; DT-62A; DT-62B; DT-71 which are peptide analogs.

We have observed that VIP (vasoactive intestinal peptide), somatostatin, substance P, and bombesin are secreted by at least some human tumor and cancer cells and that there are binding sites for these peptides on these cells. Specifically, out of a number of peptide growth regulators studied by indirect immunofluorescence, the four peptides (i.e., vasoactive intestinal peptide (VIP), somatostatin, Substance P, and bombesin) were shown to bind, to tumor cells. (Herein, the terms "peptide growth regulators", and "peptides" each refer to VIP, somatostatin, Substance P, and bombesin). It may be that there is an autocrine mechanism for cell proliferation where the peptides are secreted by tumor cells and transduce a signal through specific receptors on the same cell type leading to cell proliferation. As will be described in more detail below, the effects of the analogs of somatostatin, VIP, bombesin, Substance P on the tumor cell growth and survival were studied using different assay systems. The amino-acid sequences of the seven analogs (VIP_1, VIP_2, VIP_3, SOM_1, SOM_1, BOM_2 and SP_1) are disclosed in U.S. Application No. 08/727,679.

Code	Name	Sequence	SEQ ID NO:
VIP_1	VIP atagonist	(Lys-Pro-Arg-Arg-Pro-Tyr-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2)	SEQ ID NO:1
VIP_2	VIP receptor binding inhibitor	(Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys)	SEQ ID NO:2
VIP_3	VIP receptor antagonist	(His-Ser-Asp-Ala-Val-4-Cl-D-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Leu-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2)	SEQ ID NO: 3
SOM_1	Somatostatin analog (CTOP)	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2	SEQ ID NO: 4
SOM_2	Somatostatin analog	Ala-Gly-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-D-Cys (disulfide bridges:3-14)	SEQ ID NO: 5
BOM_1	Bombesin antagonist	D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHEt	SEQ ID NO: 6
SP_1	Substance P antagonist	D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2	SEQ ID NO: 7

As will be explained in more detail below, the combination of these seven analogs is known as MuJ-7. The analogs were synthesized manually and using a conventional peptide synthesizer. An example of a combination within the scope of the invention comprises SOM_2, VIP_1, VIP_2, VIP_3, SOM_1, BOM_1, and SP_1. A combination hereinafter referred to as MuJ-7, was prepared using the following seven

peptide analogs: (1) VIP_1, (the VIP antagonist) having a molecular weight of approximately 3464.9 and a concentration of approximately 10^{-7} M; (2) VIP_2. (the receptor binding inhibitor) having a molecular weight of approximately 1027.55 and a concentration of approximately 10^{-8}M; (3) VIP_3, (the VIP receptor antagonist) having a molecular weight of approximately 3342.09 and a concentration of approximately 10^{-8}M; (4) SOM_1 (the somatostatin analog (CTOP) having a molecular weight of approximately 1061.59 and a concentration of approximately 10^{-9}M; (5) SOM_2, (the analog of somatostatin) having a molecular weight of approximately 1637.0 and a concentration of approximately 10^{-8}M; (6) BOM_1 (the bombesin antagonist) having a molecular weight of approximately 983.55 and a concentration of approximately 10^{-8}M; and (7) SP_1 (the Substance P antagonist) having a molecular weight of approximately 1515.83 and a concentration of approximately 10^{-8}M. The preceding sentence sets forth the preferred concentrations of the seven analogs comprising MuJ-expected that MuJ-7 would be effective if the concentration of each of the seven analogs ranged from approximately 10^{-6}M to approximately 10^{-12}M.

MuJ-7 may be prepared in the following way. A stock solution of each of the seven peptide analogs is prepared with a pH of approximately 7.0 to approximately 7.4. Although sterile phosphate buffered saline was used to prepare the stock solutions for the testing described below, other diluents may be used such as RPMI 1649, buffered saline, isotonic NaCl, Ringer's solution, water (for injection) distilled water, polyethylene glycol (near or in water), 2% Tween in water, dimethylsulfoxide to 50% in water, propylene glycol (neat or in water), balanced salt solution, glycerol, and other conventional fluids that are suitable for intravenous administration to obtain a pH in the range of approximately 7.0 to approximately 7.4 for each stock solution, the pH can be adjusted by using 1 N HCl for lowering the pH or 1 N NaOH for raising the pH, although the conventional agents for adjusting the pH can be used, the concentration of the peptide analog in each stock solution is approximately 1^{-3}M.

Aliquots of the seven peptides analogs are mixed together such that the MuJ-7 formulation contained approximately equal weights of each of the seven peptide analogs. In MuJ-7, approximately, the concentration of VIP_1, is 10^{-7}M; the concentration of VIP_2 is 10^{-8}M; the concentration of VIP_3 is 10^{-8}M; the concentration

of SOM1 is 10^{-9}M; the concentration of SOM_2 is 10^{-8}M; the concentration of BOM1 is 10^{-8}M; and the concentration of SP_1 is 10^{-8}M. In one exemplary embodiment, the pH of the MuJ-7 solution may range from about 7.0 to 7.4. To obtain a pH in this range, the pH can be adjusted by using 1 N NC1 for lowering the pH or 1 N NaOH for raising the pH, although other conventional agents for adjusting the pH can be used.

In addition, a number of novel peptides also exhibited an antiangiogenic effect. These peptides are:

DT-11	Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH	(SEQ ID NO: 8)
DT-12	D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH	(SEQ ID NO: 9)
DT-13	Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH	(SEQ ID NO: 10)
DT-14	Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 11)
DT-15	Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 12)
DT-16	D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 13)
DT-18	Aib-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH	(SEQ ID NO: 14)
DT-19	D-Leu-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH	(SEQ ID NO: 15)
DT-23	D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2	(SEQ ID NO: 16)
DT-24	D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2	(SEQ ID NO: 17)
DT-26	D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2	(SEQ ID NO: 18)
DT-27	D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2	(SEQ ID NO: 19)
DT-31	Aib-Met-Gln-Trp-Phe-Aib-NH_2	(SEQ ID NO: 20)
DT-33	D-Leu-Met-Gln-Trp-Phe-Aib-NH_2	(SEQ ID NO: 21)
DT-34	D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2	(SEQ ID NO: 22)
DT-62A	Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond)	(SEQ ID NO: 23)
DT-62B	Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys	(SEQ ID NO: 24)
DT-71	D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2	(SEQ ID NO: 25)

Note: - Aib = α-Aminoisobutyric acid, Deg = α, α Diethyl glycine

Any one or two amino acids in the sequence Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys-OH (SEQ ID NO: 2) may be replaced by Aib or Deg.

Any one or two amino acids in the sequence D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO: 16) may be replaced by Aib or Deg.

Any one or two amino acids in the sequence Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 20) may be replaced by Aib or Deg.

Any one or two amino acids in the sequence Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 disulphide bond) (SEQ ID NO: 23) may be replaced by Aib or Deg.

Any one or two amino acids in the sequence D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25) may be replaced by Aib or Deg.

Aib and Deg are defined above.

Any one or two amino acids in the sequence Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 8) may be replaced by its respective D-amino acid.

Any one or two amino acids in the sequence D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO: 16) may be replaced by its respective D-amino acid.

Any one or two amino acids in the sequence Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 20) may be replaced by its respective D-amino acid.

Any one or two amino acids in the sequence Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (SEQ ID NO: 23) may be replaced by its respective D-amino acid.

Any one or two amino acids in the sequence D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25) may be replaced by its respective D-amino acid.

MuJ-7 was tested against primary tumor cells of human colon adenocarcinoma, and each of the peptide analogs comprising MuJ-7 was tested individually against human colon adenocarcinoma cells. A three day MTT cytotoxicity assay was performed as described in U.S. Patent Application No. 08/727,679. The percent killing achieved by individual peptides was in the range of 54 to 79% while percent killing achieved by MuJ-7 was 94%.

Five different subcombinations of seven peptide analogs comprising MuJ-7 were tested against human colon adenocarcinoma cells. Each subcombination was tested by performing a one day MTT cytotoxicity assay as described in U.S. Patent Application No. 08/727,679. The percent killing achieved by the

subcombinations was in the range of 64.7% to 94.9%.

EXAMPLE 1

ECV.3O4 cells collected at exponential growth phase were resuspended in medium (3.3×10^6 cells/ml in RPMI 1640 containing 10% FBS). 150µl of medium was added to the wells of a 96-well tissue culture plate (Nunc, Denmark) followed by 30µl of cell suspension. The plate was left in incubator (37°C, 5% CO_2) overnight. Each of the seven peptides of the combination MuJ-7 were added at three molar concentrations of 10^{-7}M, $1O^{-8}$M and 10^{-9}M. 20µl of MuJ-7 at three concentrations of N/10, N and 10N was added to marked wells of the 96-well plate. The value of N for each of the individual peptides was 10^{-8}M for VIP_2, BOM_1, SP_1, VIP_3, and SOM_2 and 10^{-7}M for VIP_1, and 10^{-9}M for SOM_1. Each concentration was plated in triplicate. 20µl of medium alone was added to control wells while wells without cells served as blanks. A total volume of 200µl was ensured in each well and the plate was left in an incubator (37°C, 5% CO_2). After 72 hours of incubation an MTT assay was performed and percentage inhibition in proliferation of treated cells was calculated with respect to control cells. Results are given in Table I.

TABLE 1

Percent inhibition in proliferation of endothelial cells on treatment with different concentrations of MuJ-7 and its constituent peptides.

Cell line	Percent Inhibition Proliferation (%)																							
	VIP_2			BOM_1			SP_1			VIP_1			VIP_3			SOM_2			SOM_1			MuJ-7		
	-7	-8	-9	-7	-8	-9	-7	-8	-9	-7	-8	-9	-7	-8	-9	-7	-8	-9	-7	-8	-9	N/10	N	10N
EaHy.926	32	28	42	38	31	32	49	32	37	25	24	23	21	9	24	36	38	25	36	36	22	36	44	38
ECV.304	24	25	22	21	22	19	18	24	22	15	17	16	21	25	22	20	19	17	20	20	19	24	31	28

EXAMPLE 2

ECV304 cells collected at exponential growth phase were resuspended in medium (3.3 x 10^6 cells/ml in RPMI 1640 containing 10% FBS). 150µl of medium was added to the wells of a 96-well tissue culture plate (Nunc, Denmark) followed by 30µl of cell suspension. The plate was left in incubator (37°C, 5% CO_2) overnight. Novel peptides shown below were incubated with cells at concentration range of 10^{-7} to 10^{-9} M (except DT71 at concentration range 10^{-8} to 10^{-10} M). Each concentration was plated in triplicates. 20µl of medium alone was added to control wells while wells without cells served as blanks. A total volume of 200µl was ensured in each well and plate was left in incubator (37°C, 5% CO_2). After 72 hours of incubation an MTT assay was performed and percentage inhibition in proliferation of treated cells was calculated with respect to control cells. Results are given in Table II.

SEQUENCE LISTING OF NEW PEPTIDE ANALOGS

DT-11	Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH	(SEQ ID NO: 8)
DT-12	D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH	(SEQ ID NO: 9)
DT-13	Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH	(SEQ ID NO: 10)
DT-14	Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 11)
DT-15	Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 12)
DT-16	D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 13)
DT-18	Aib-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH	(SEQ ID NO: 14)
DT-19	D-Leu-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH	(SEQ ID NO: 15)
DT-23	D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2	(SEQ ID NO: 16)
DT-24	D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2	(SEQ ID NO: 17)
DT-26	D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2	(SEQ ID NO: 18)
DT-27	D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2	(SEQ ID NO: 19)
DT-31	Aib-Met-Gln-Trp-Phe-Aib-NH_2	(SEQ ID NO: 20)
DT-33	D-Leu-Met-Gln-Trp-Phe-Aib-NH_2	(SEQ ID NO: 21)
DT-34	D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2	(SEQ ID NO: 22)
DT-62A	Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond)	(SEQ ID NO: 23)

DT-62 Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (SEQ ID NO: 24)

DT-71 D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25)

Note: -Aib = α-Aminoisobutyric acid, Deg =α, α Diethyl glycine

TABLE 2

COMPOUND	PERCENT INHIBITION OF PROLIFERATION		
	10^{-7}M	10^{-8}M	10^{-9}M
DT-11	16.7	18.7	19.9
DT-12	20.3	16.1	15.5
DT-13	22.6	20.3	18.4
DT-14	17.0	6.0	20.5
DT-15	20.4	13.2	20.0
DT-16	24.0	24.7	37.9
DT-18	23.3	10.9	14.4
DT-19	11.9	7.3	21.0
DT-23	3.5	17.2	30.0
DT-24	6.4	9.8	10.7
DT-26	0	0	0
DT-27	14.3	11.9	6.7
DT-31	1.3	14.1	6.1
DT-33	8.7	15.8	13.9
DT-34	12.1	15.6	14.3
DT-62A	11.7	20.1	25.7
DT-62B	14.0	3.8	12.8
	$10.^{-8}$M	10^{-9}M	10^{-10}M
DT-71	14.6	12.1	9.4

EXAMPLE 3

Polycarbonate filter transwell inserts (24 well size) with 8 μm pores (Nunc, Denmark) were used for the migration assay. ECV304 (10^4 cells/200 μl

DMEM containing 0.1 %BSA) was added to the upper chamber. The lower chamber contained 600 µl of DMEM with 0.1% BSA. Individual peptides of the composition MuJ-7 at six different concentrations each of N/100, N/10, N, 10N, 100N and 1000N were added directly to the upper well and the plate incubated at 37°C for 24 hours. The cells migrated to the lower chamber were viewed randomly at five different phase-contrast microscopic fields and the total number of cells counted using Video Pro 32 Image Analysis system. Percent inhibition in migration was determined with reference to the Control. Similarly, five different concentrations of N/100, N/10, N, 10N, 100N and 1000N of MuJ-7 were added directly to the upper well and the plate incubated at 37°C for 24 hours. The cells migrated to the lower chamber were counted as described above. The molarity representing N for each peptide is given in Table No. 3.

TABLE 3

Percent inhibition in migration of endothelial cells ECV.304 across a 8µ filter on treatment with various concentrations of MuJ-7 and its constituent peptides.

Peptide	Percent inhibition in migration (%)					
	N/100	N/10	N	10N	100N	1000N
VIP_2	0.00	91.75	89.05	88.15	92.90	0.00
BOM_1	0.00	18.25	32.85	24.10	63.95	0.00
SP_1	13.97	94.10	90.50	92.65	78.10	18.10
VIP_1	37.53	52.60	69.40	62.60	66.52	0.00
VIP_3	0.00	32.80	92.45	23.90	11.62	0.00
SOM_2	7.21	90.70	92.30	89.75	82.15	58.00
SOM_1	84.27	78.50	88.11	88.75	73.73	25.00
MuJ-7	0.00	93.39	95.69	97.30	21.6	-

where the value of N for each peptide is 10^{-8} M for VIP_2, BOM_1, SP_1, VIP_3, and SOM_2 and 10^{-7}M for VIP_1, and 10^{-9}M for SOM_1.

EXAMPLE 4

Polycarbonate filter trasnwell inserts (24 well size) with 8 μm pores (Nuno, Denmark) were used for the migration assay. ECV304 (10^4 cells/200 μl DMEM containing 0.1% BSA) was added to the upper chamber. The lower chamber contained 600 μl of DMEM with 0.1% BSA. Individual peptides at optimum concentration (as indicated in Table 4) were added directly to the upper well and the plate incubated at 37°C for 24 hours. The sequence of each of the peptides added are given in Figure 1. The cells migrated to the lower chamber were viewed randomly at five different phase-contrast microscopic fields and the total number of cells counted using Video Pro 32 Image Analysis system. Percent inhibition in migration was determined with reference to Control.

TABLE 4

Percent inhibition in migration of endothelial cells ECV304

across a 8μ filter on treatment with analogs shown in Figure 1

COMPOUND	PERCENT INHIBITION MIGRATION
DT-11(-8M)	82.5
DT-12 (-8M)	58.7
DT-13 (-8M)	89.7
DT-14 (-8M)	77.3
DT-15 (-8M)	73.2
DT-16 (-8M)	90.07
DT-18 (-8M)	73.2
DT-19 (-8M)	80.4
DT-23 (-8M)	0
DT-24 (-8M)	0
DT-26 (-8M)	0
DT-27 (-8M)	0
DT-31 (-8M)	0
DT-33 (-8M)	41.4
DT-34 (-8M)	53.6
DT-62A (-8M)	62.2
DT-62B (-8M)	8.5
DT-71 (-9M)	68.2

EXAMPLE 5

Polycarbonate filter transwell inserts (24 well size) with 8 μm pores (Nunc, Denmark) were used for the migration assay as described in the previous example. ECV304 (10^4 cells/200 μl DMEM containing 0.1% BSA) was added to the upper chamber.

The lower chamber contained 600 μl of DMEM with 0.1% BSA. Ten different subcombinations of MuJ-7 listed in Table 5 were tested for inhibition of migration of endothelial cells across the filter as described previously. None of the ten subcombinations caused percent inhibition of migration greater than MuJ-7 and was in the range of 11.3 to 69.3% (Table 6).

TABLE 5

Ten combinations of MuJ-7 with different ratios of its constituent peptides.

Combination	VIP_1	VIP_2	VIP_3	SOM_1	SOM_2	BOM_1	SP_1
MuJ-7	-7	-8	-8	-9	-8	-8	-8
1	-8	-8	-8	-9	-8	-8	-6
2	-6	-8	-8	-9	-8	-8	-10
3	-7	-9	-8	-9	-8	-6	-8
4	-7	-7	-8	-9	-8	-10	-8
5	-7	-6	-7	-9	-8	-8	-8
6	-7	-10	-9	-9	-8	-8	-8
7	-5	-8	-8	-8	-8	-8	-7
8	-9	-8	-8	-9	-8	-8	-9
9	-7	-8	-6	-9	-8	-7	-8
10	-7	-8	-10	-9	-8	-9	-8

TABLE 6

Percent inhibition in migration of endothelial cells ECV.304 across a 8μ filter on treatment with ten different combinations of MuJ-7

Subcombination	% Inhibition Migration
MuJ-7	95.7
1	38.6
2	69.3
3	19.3
4	50.0
5	11.3
6	28.4
7	13.6
8	37.5
9	32.9
10	25.0

EXAMPLE 6

Matrigel (350 µl) was placed into each well of a 24-well culture plate at 4°C and was allowed to polymerize by incubation at 37°C for 30 min. ECV304 ($1.5x10^4$) were seeded on the Matrigel in 500 µl DMEM supplemented with 10% FBS. MuJ-7 at four different concentrations of 0.25x, 0.5x, x, & 5.0x were added and the plate incubated at 37°C for 24 hour. "x" denotes normal concentration of MuJ-7 where molar concentration of individual peptides was 10^{-8} M for VIP_2, BOM_1, SP_1, VIP_3 and SOM_2 and 10^{-7} M for VIP_1 and 10^{-9} M for SOM_1. Tube like structures formed, the area of which was individually counted at five different phase-contrast microscopic fields using Video Pro 32 Image Analysis system. Percent inhibition in tube area was determined with reference to untreated cells.

TABLE 7

Percent inhibition in Tube-like-structure (TLS) area on treatment with various concentrations of MuJ-7.

Concentration of MuJ-7	Percent in tube area
0.25x	28.4
0.5x	39.3
x	47.8
5.0x	58.1

EXAMPLE 7

Matrigel (350 µl) was placed into each well of a 24-well culture plate at 4°C and was allowed to polymerize by incubation at 37°C for 30 min. ECV304 (1.5 x 10^4) were seeded on the Matrigel in 500 µl DMEM supplemented with 10% FBS. New analogs of peptides as shown in Figure 1 were added at optimum concentration as denoted in Table 8, and the plate incubated at 37°C for 24 hours. Tube like structures formed, the area of which was individually counted at five different phase-contrast microscopic fields using Video Pro 32 Image Analysis system. Percent inhibition in tube length was determined with reference to untreated cells.

TABLE 8

PEPTIDE	SPROUTING	INTUSSUSCEPTION	TUBE FORMATION	INHIBITION OF TUBE LENGTH
DT-10 (-8M)	+	+	+	59.5
DT-11 (-8M)	++	+	+	3.6
DT-12 (-8M)	++	+	++	17.1
DT-13 (-8M)	++	++	++	16.2
DT-14 (-8M)	++	+	+	24.3
DT-15 (-8M)	++	+	++	0
DT-16 (-8M)	++	+	+	18.9
DT-18 (-8M)	++	++	++	0
DT-19 (-8M)	++	+	+	37.8
DT-23 (-8M)	+	+	+	53.1
DT-24 (-8M)	++	+	+	16.2

DT-26 (-8M)	++	++	++	0
DT-27 (-8M)	+	+	+	29.7
DT-31 (-8M)	+	+	+	17.1
DT-33 (-8M)	++	++	++	13.5
DT-34 (-8M)	++	++	++	0
DT-62A (-8M)	++	+	++	44.1
DT-62B (-8M)	++	++	++	0
DT-71 (-9M)	++	+	++	9.9

EXAMPLE 8

Matrigel (350 µl) was placed into each well of a 24-well culture plate at 4°C and was allowed to polymerize by incubation at 37°C for 30 min. ECV304 (1.5×10^4) were seeded on the Matrigel in each of the 24 wells. 500µl DMEM supplemented with 10% FBS and was added to 8 wells. These wells served as controls. Culture supernatants of primary tumor cells of human colon adenocacinoma from a confluent culture in log phase of growth was filtered through a 0.22 µ filter and its pH adjusted to 7.4 with sodium bicarbonate. 500 µl of this supernatant was added to another 8 wells, containing ECV304 (1.5×10^4) cells seeded on Matrigel. To the remaining 8 wells, MuJ-7 was added and incubated at 37°C for 24 hours. Five different phase-contrast microscopic fields (4X) were reviewed and total tube length of the tube-like-structures (TLS) measured using Video Pro 32 Image Analysis system. Percent inhibition of TLS was calculated with reference to Controls. In controls, although sprouting was visible at day 1, intussusception was still missing. However, endothelial cells treated PTC culture supernatant showed enhanced sprouting as well as intussusception leading to completion of tube formation. This clearly suggests pro-angiogenic activity of factors in culture supernatant of primary tumor cells of human colon adenocarcinoma. Treating PTC culture supernatant treated endothelial cells with MuJ-7 resulted in complete inhibition of intussusception and a significant decrease in sprouting. This indicates the ability of MuJ-7 to inhibit two key steps of a new blood vessel formation, i. e., sprouting and angiogenesis. A qualitative representation of the effect is given in Table 9.

TABLE 9

Qualitative representation of the effect of MuJ-7 on Tube-like-structure activity of PTC culture supernatant stimulated endothelial cells.

	Untreated	PTC culture supernatant stimulated	MuJ-7 treated
Sprouting	++	+++	+
Intussusception	+	++	-
Tube formation	+	++	-

EXAMPLE 9

Matrigel (350 µl) was placed into each well of a 24-well culture plate at 4°C and was allowed to polymerize by incubation at 37°C for 30 min. ECV304 (1.5×10^4) were seeded on the Matrigel in 500 µl DMEM supplemented with 10% FBS. Individual peptides of the composition MuJ-7 at three different concentrations each of N/10, N, and 10N were added and the plate incubated at 37°C for 24 hours. Tube like structures formed, the length of which were individually counted at five different phase-contrast microscopic fields using Video Pro 32 Image Analysis system. Percent inhibition in tube length was determined with reference to Control. Similarly, three different concentrations of N/10, N, and 10N of MuJ-7 were added to the well and the plate incubated at 37°C for 24 hours. Percent inhibition in tube length was determined with reference to Control as described previously (Table 10).

TABLE 10

Percent inhibition in Tube-like-structure (TLS) length on treatment with various concentrations of MuJ-7 and its constituent peptides.

Peptide	Percent inhibition in TLS activity (%)		
	N/10	N	10N
VIP_2	0.0	1.0	3.3
BOM_1	0.0	0.0	2.9
SP_1	14.4	8.9	3.0
VIP_1	14.9	11.7	13.1
VIP_1	8.5	22.1	17.9
SOM_2	1.0	16.5	16.8
SOM_2	7.0	15.1	16.7
MuJ-7	6.9	15.6	22.27

where the value of N for each peptide is 10^{-8}M for VIP_2, BOM_1, SP_1, VIP_3, and SOM_2 and 10^{-7}M for VIP_1, and 10^{-9}M for SOM_1.

EXAMPLE 10

Matrigel (350 µl) was placed into each well of a 24-well culture plate at 4°C and was allowed to polymerize by incubation at 37°C for 30 min. ECV304 (1.5×10^4) were seeded on the Matrigel in each of the 24 wells. 500 µl DMEM supplemented with 10% FBS was added to 8 wells. These wells served as controls. Culture supenatants of primary tumor cells of human colon adenocarcinoma from a confluent culture in log phase of growth was filtered through a 0.22 µ filter and its pH adjusted to 7.4 with sodium bicarbonate. 500 µl of this supematant was added to another 8 wells contains ECV304 (1.5×10^4) cells seeded on Matrigel. To the remains 8 wells, MuJ-7 was added and incubated at 37°C for 24 hours. Five different phase-contrast microscopic fields (4X) were viewed and total tube length of the tube-like-structures (TLS) measured using Video Pro 32 Image Analysis system. Percent inhibition of TLS was calculated with reference to Controls. In controls, 76.9% of TLS were in the range of 50 - 69 µm and remaining were between 69 - 107 µm. There were no structures greater than 107 µm. In PTC stimulated wells, the percentage of TLS in range of 50 - 69 µm reduced to 50.0% while 27.3% were in the range of 69 - 107 µm. It is interesting to note that there were approximately 18.0% TLS in the range of 107 - 183 µm and 4.55% TLS in 221 - 240 µm. Hence

there was a significant increase in the length of TLS in PTC supernatant stimulated cells as compared to the unstimulated controls. This clearly suggests pro-angiogenic activity of factors in culture supematant of primary tumor cells of human colon adenocarcinoma. In the third group of wells, which were treated with MuJ-7, 81.25% TLS were in the range of 50 - 69 μm and 18.75% in the range of 69 - 88 μm (Table 6). This reversal of TLS activity to control level on treatment with MuJ-7 clearly suggests anti angiogenic activity of MuJ-7.

TABLE 11

Percent inhibition in TLS length of PTC culture supernatant endothelial cells treated with MuJ-7.

Tube length Range (in microns)	Controls	PTC culture supernatant stimulated	MuJ-7 treated
50 - 69	76.92	50.00	81.25
69 - 88	7.69	4.55	18.75
88 - 107	15.38	22.73	0.00
107 - 126	0.00	4.55	0.00
126 - 145	0.00	9.09	0.00
145 - 164	0.00	0.00	0.00
164 - 183	0.00	4.55	0.00
183 - 202	0.00	0.00	0.00
202 - 221	0.00	0.00	0.00
221 - 240	0.00	4.55	0.00

Neo vascularization, an essential event for the growth of solid tumors, is regulated by a number of angiogenic factors. One such factor, vascular endothelial growth factor (VEGF), is considered to exert a potent angiogenic activity. Recent studies have demonstrated that the VEGF is strongly expressed in several human solid tumors, and its expression is correlated with the density of microvessels in tumors of the kidney, breast, brain, colon, stomach, lung, oesophagus and liver. Tumor vascularity was correlated directly with VEGF production by the tumor, indicating

that VEGF production is a relevant factor in determining angiogenesis in primary tumor. VEGF enhances the destruction of extracellular matrix by increasing urokinase-type plasminogen activator and urokinase-type plasminogen receptors on endothelial cells which degrade extracellular matrix and allow cancer cell invasion.

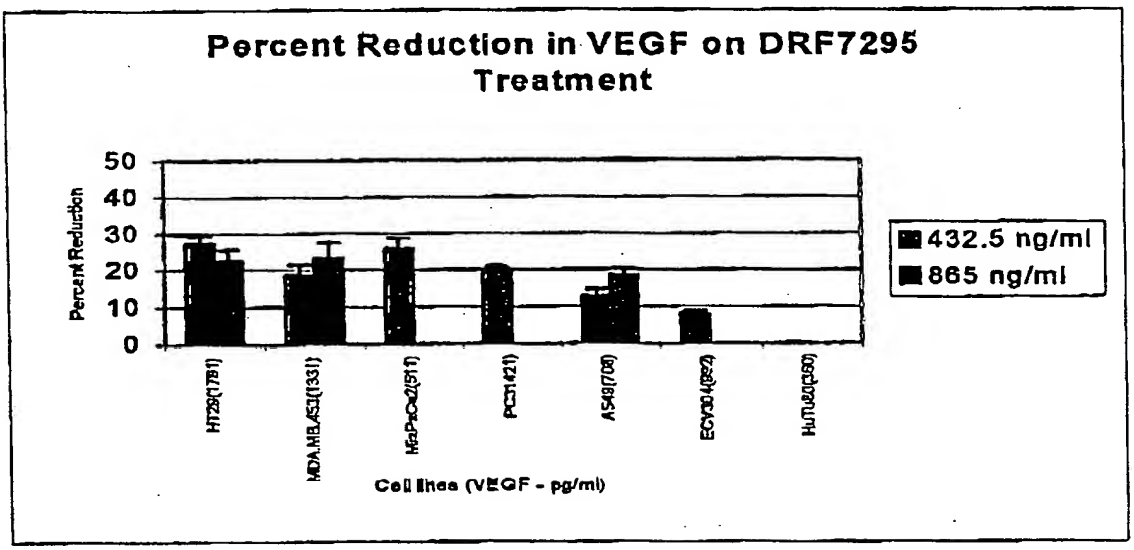
METHOD

Human adenocarcinoma cells such as MiaPaCa (Pancreas), PC3(Prostate), HT29(Colon), HuTu80 (Stomach), MDA.MB.453 (BREAST), PTC (Colon), A549 (Lung) and human endothelial cells (ECV304) were plated at the density of 8-10x10^5 cells per 2 ml in a six well plate. After an overnight incubation of cells at 37°C, DRF-7295 (1X & 2X) was added to the wells. The untreated wells served as controls. The plates were incubated for 4 hours at 37°C. The medium was collected from all the wells (control and treated) and spun down at 2000 r.p.m to remove the cellular material. The supernatant was collected and use for ELISA (Quantikine human VEGF, R&D System). The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and VEGF presents was bound by the immobilized antibody. After washing away the unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of VEGF bound in the initial step. The optical density of each well was determined within 30 minutes using a microplate reader set to 450nm and reference wavelength at 540nm.

RESULTS

The cell showed high levels of secretion of VEGF, which was inhibited by the addition of DRF7295. However, no significant difference was seen in inhibition at 1X and 2X concentrations of the drug. The results of the assay are summarized in the following Table and depicted in the figure below.

S.No	Cell line	Normal level of VEGF (pg/ml)	Percent reduction in VEGF levels on treatment with DRF7295	
			432.5 ng/ml	865 ng/ml
1	HT29 (colon)	1791 ± 34	27.2 ± 2.1	22.3 ± 3.2
2	MDA.MB.453 (breast)	1331 ± 28	18.5 ± 2.7	22.9 ± 4.5
3	MiaPaCa2 (pancreas)	511 ± 17	25.4 ± 3.2	ND
4	PC3 (prostate)	1421 ± 47	19.1 ± 1.7	ND
5	A549 (lung)	708 ± 18	12.8 ± 1.9	18.4 ± 1.9
6	HuTu80 (stomach)	360 ± 16	8.0 ± 0.9	ND
7	ECV304 (endothelial)	992 ± 27	0	0



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CLAIMS

1. A therapeutically effective composition of peptide SOM_2 and at least four peptides selected from the group consisting of VIP_1, VIP_2, VIP_3, SOM_1, BOM_1, AND SP_1, for treatment and/or prevention of angiogenesis in humans and animals.

2. A composition as claimed in claim 1, comprising a therapeutically effective combination of VIP_1, VIP_2, SOM_1, SOM_2, AND BOM_1.

3. A composition as claimed in claim 1, comprising a therapeutically effective combination of VIP_1, VIP_2, VIP_3, SOM_1, BOM_1, AND SP_1,

4. A composition as claimed in claim 3, wherein the concentration of VIP_1, is about 10^{-7}M, the concentration of VIP_2, is about 10^{-8}M, the concentration of VIP_3 is about 10^{-8}M, the concentration of SOM_1, is about 10^{-9}M, the concentration of SOM_2 is about 10^{-7}M, the concentration of BOM_1, is about 10^{-8}M, and the concentration of SP_1, is about 10^{-8}M.

5. A pharmaceutical composition as claimed in claim 3, wherein the molar ratio of VIP_1: VIP_2: VIP_3: SOM_1: SOM_2: BOM_1: SP_1 is about 1.0:0.1:0.1:0h01:0.1:0.1:0.1.

6. A composition as claimed in any of claims 1-5, further comprising a pharmaceutically acceptable carrier, diluent or solvent.

7. A method of treatment of angiogenesis in human beings or animals which comprises administering a therapeutically effective dose of a composition comprising SOM_2 and at least four peptides selected from the group consisting of VIP_1, VIP_2, VIP_3, SOM_1, BOM_1, and SP_1.

8. A method according to claim 7, wherein the composition comprises VIP_1, VIP_2, VIP_3 SOM_1, SOM_2, BOM_1, and SP_1.

9. A method according to claim 8, wherein the composition concentration of VIP_1, is about 10^{-7}M, the concentration of VIP_2 is about 10^{-8}M, the concentration of VIP_2 is about 10^{-8}M, the concentration of SOM_1, is about 10^{-9}M, the concentration of SOM_2 is about 10^{-7}M, the concentration of BOM_1, is about 10^{-8}M, and the

concentration of SP_1, is about 10^{-8}M.

10. A method of treatment of angiogenesis in human beings or animals which comprises administering a therapeutically effective dose of a peptide selected from the group consisting of VIP_1, VIP_2, VIP_3, SOM_1, SOM_2, BOM_1, and SP_1.

11. A composition comprising a peptide selected from:

Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH	(SEQ ID NO: 8)
D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH	(SEQ ID NO: 9)
Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH	(SEQ ID NO: 10)
Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 11)
Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 12)
D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH	(SEQ ID NO: 13)
Aib-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH	(SEQ ID NO: 14)
D-Leu-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH	(SEQ ID NO: 15)
D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2	(SEQ ID NO: 16)
D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2	(SEQ ID NO: 17)
D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2	(SEQ ID NO: 18)
D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2	(SEQ ID NO: 19)
Aib-Met-Gln-Trp-Phe-Aib-NH_2	(SEQ ID NO: 20)
D-Leu-Met-Gln-Trp-Phe-Aib-NH_2	(SEQ ID NO: 21)
D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2	(SEQ ID NO: 22)
Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond)	(SEQ ID NO: 23)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys or (SEQ ID NO: 24)

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25)

wherein Aib = α-Aminoisobutyric acid, Deg = α, α Diethyl glycine.

12. A composition as claimed in claim 11, further comprising a pharmaceutically acceptable carrier, diluent or solvent.

13. A method of treatment of angiogenesis in human beings or animals which comprises administering a therapeutically effective dose of a peptide selected from:

Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 8)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 9)

Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH (SEQ ID NO: 10)

Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 11)

Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 12)

D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 13)

Aib-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 14)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 15)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO: 16)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2 (SEQ ID NO: 17)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2 (SEQ ID NO: 18)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2 (SEQ ID NO: 19)

Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 20)

D-Leu-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 21)

D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2 (SEQ ID NO: 22)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond) (SEQ ID NO: 23)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys and (SEQ ID NO: 24)

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25)

wherein -Aib = α-Aminoisobutyric acid, Deg =α, α Diethyl glycine.

14. The method according to claim 13, wherein the angiogenesis is a result of adenocarcinoma cancers of the colon, breast, lung, prostate, kidney, leukemias or lymphomas.

15. Use of a peptide selected from:

Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 8)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 9)

Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH (SEQ ID NO: 10)

Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 11)

Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 12)

D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 13)

Aib-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 14)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 15)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO: 16)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2 (SEQ ID NO: 17)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2 (SEQ ID NO: 18)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2 (SEQ ID NO: 19)

Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 20)

D-Leu-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 21)

D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2 (SEQ ID NO: 22)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond) (SEQ ID NO: 23)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys or (SEQ ID NO: 24)

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25)

wherein Aib = α-Aminoisobutyric acid, Deg =α, α Diethyl glycine for the prevention or treatment of angiogenesis in human beings or animals.

16. A medicine for treatment of angiogenesis in human beings or animals which comprises a peptide selected from the group consisting of:

Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 8)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 9)

Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH (SEQ ID NO: 10)

Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 11)

Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 12)

D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 13)

Aib-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 14)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 15)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO: 16)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2 (SEQ ID NO: 17)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2 (SEQ ID NO: 18)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2 (SEQ ID NO: 19)

Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 20)

D-Leu-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 21)

D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2 (SEQ ID NO: 22)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond) (SEQ ID NO: 23)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys and (SEQ ID NO: 24)

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25)

wherein -Aib = α-Aminoisobutyric acid, Deg =α, α Diethyl glycine.

17. Use of a peptide selected from:

Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 8)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH (SEQ ID NO: 9)

Leu-Met-Tyr-Pro-Thr-D-Tyr-Leu-Lys-OH (SEQ ID NO: 10)

Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 11)

Leu-Met-D-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 12)

D-Leu-Met-Tyr-Pro-Thr-Tyr-D-Leu-Lys-OH (SEQ ID NO: 13)

Aib-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 14)

D-Leu-Met-Tyr-Pro-Thr-Tyr-Deg-Lys-OH (SEQ ID NO: 15)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO: 16)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH_2 (SEQ ID NO: 17)

D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile-NH_2 (SEQ ID NO: 18)

D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile-NH_2 (SEQ ID NO: 19)

Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 20)

D-Leu-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 21)

D-Arg-Pro-Lys-Pro-Aib-Gln-D-Trp-Phe-D-Trp-Aib-Leu-NH_2 (SEQ ID NO: 22)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond) (SEQ ID NO: 23)

Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys or (SEQ ID NO: 24)

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2 (SEQ ID NO: 25)

wherein Aib = α-Aminoisobutyric acid, Deg =α, α Diethyl glycine for the manufacture of a medicament for the prevention or treatment of angiogenesis in human beings or animals.

18. A peptide comprising the sequence Aib-Met-Tyr-Pro-Thr-Tyr-Aib-Lys-OH, wherein Aib represents α-aminoisobutyric acid (SEQ ID NO: 8) or a sequence wherein any one or two the of the amino acids is replaced by its respective D-amino acid; D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2, wherein Aib represents α-amino-isobutyric acid (SEQ ID NO: 16) or a sequence wherein any one or two of the amino acids is replaced by its respective D-amino acid; Aib-Met-Gln-Trp-Phe-Aib-NH_2, wherein Aib represents α-aminoisobutyric acid (SEQ ID NO: 20) or a sequence wherein any one or two of the amino acids is replaced by its respective D-amino acid; Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond) wherein Aib represents α-aminoiso-butyric acid (SEQ ID NO: 23) or a sequence wherein any one or two of the amino acids is replaced by its respective D-amino acid or D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2, wherein Aib represents α-aminoisobutyric acid (SEQ ID NO: 25) or a sequence wherein any one or two of the amino acids is replaced by its respective D-amino acid.

19. A peptide selected from: Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys-OH (SEQ ID NO: D-Phe-Gln-Trp-Aib-Val-Gly-His-Leu-NH_2 (SEQ ID NO: 16); Aib-Met-Gln-Trp-Phe-Aib-NH_2 (SEQ ID NO: 20); Ala-Aib-Cys-Lys-Asn-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-Ser-Cys (3-14 Disulphide bond) (SEQ ID NO: 23); or D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Aib-Thr-NH_2, (SEQ ID NO: 25) wherein one or two amino acids of any of the sequences is replaced by Aib or Deg where Aib is 2-aminoisobutyric acid and Deg is α,α-diethyl glycine.

20. Use of a peptide according to claim 18, for treatment of angiogenesis.

21. Use of a peptide according to claim 19, for treatment of angiogenesis.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/03559

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 38/00, 38/02; C07K 5/00, 7/00
US CL :514/12, 14, 16; 530/311, 324, 327, 328, 329
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 14, 16; 530/311, 324, 327, 328, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,552,520 A (KIM et al) 03 September 1996, col. 5, lines 29-44, col. 22, lines 14-31 and claims 14-28.	1-10
Y	US 5,410,019 A (COY et al) 25 April 1995, col. 3, lines 61 to col. 9, lines 4.	1-10
Y	US 5,217,955 A (BOGDEN et al) 08 June 1993, col. 3, lines 27 to col. 9, lines 41.	1-10
Y	US 5,565,424 A (GOZES et al) 15 October 1996, col. 3, lines 45 to col. 6, lines 64 and claims 25-26.	1-10
A	WO 96 39161 A1 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 12 December 1996, pages 3-5 and 14-15.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
14 JUNE 2000

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/03559

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DANESI et al. The Effects of the Somatostain Analog Octreotide on Angiogenesis In Vitro . Metabloism. August 1996, Vol. 45, No. 8, Suppl. 1, pages 49-50, especially page 50, last paragraph.	1-10
A	WOLTERING et al. Somatostain Analogues Inhibit Angiogenesis in the Chick Chorioallantoic Membrane. Journal of Surgical Research. 1991, Vol. 50, No. 3, pages 245-251, especially pages 249-250.	1-10
A	HANAHAN et al. Patterns and Emerging Mechanisms of the Angiogenic Switch during Tmorigenesis. Cell. 09 August 1996, Vol. 86, pages 353-364, especially pages 358-360.	1-21

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, MEDLINE, EMBASE, WPIDS, BIOSIS, MPSRCH
search terms: peptide or polypeptide or protein (5l) angiogenesis, vasactive intestinal peptide or VIP or VIP1 or VIP antagonist or VIP2 or VIP receptor binding inhibitor or VIP3 or VIP receptor antagonist, somatostain analog or SOM1 or CTOP or SOM2, bombesin antagonist or BOM1, substance P antagonist or SP1, MuJ-7, treat? or inhibit? or prevent? or therpeutic?